



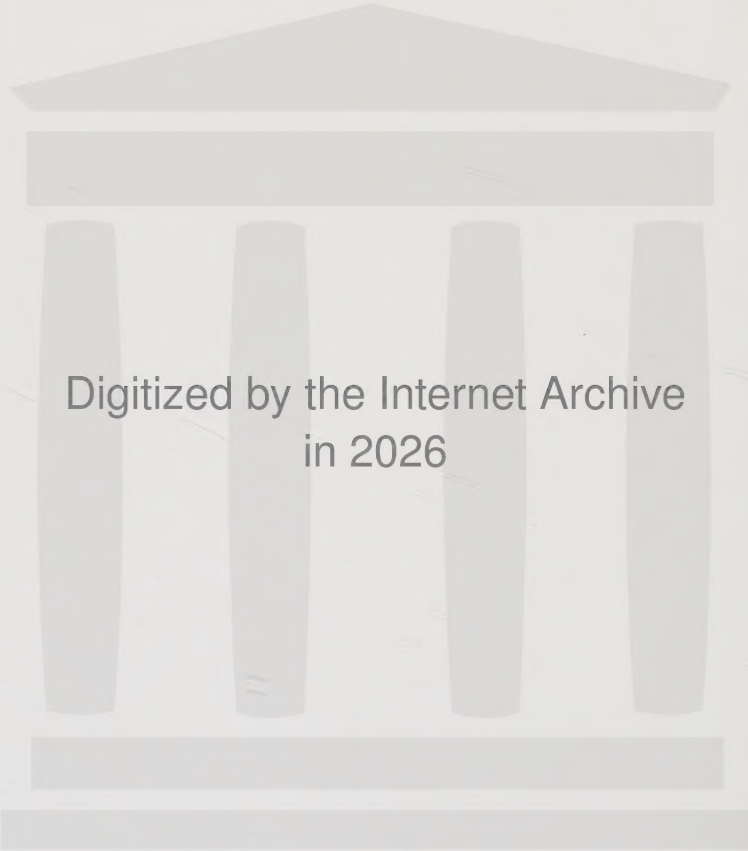
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CELL AGEING AND LONGEVITY: TOWARDS A MODEL BASED ON EXPERIMENTAL STUDIES IN MAMMALIAN CELL CULTURES

Dan Röhme

Lund 1981.

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Dan Röhme (Sm)

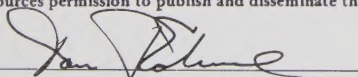
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Abstract This investigation focuses on comparative analysis of mammalian longevity and the constitutional factors on which this may rely. In particular, emphasis has been placed on (i) cellular life-span in culture, (ii) constitutional factors (especially the cell fusion sensitivity (FS) potential) related to the cellular life-span and (iii) the organization of the chromosome as revealed by premature chromosome condensation (PCC). Evidence is presented to favour the concept that: (1) The longevity of a mammalian species is basically related to the longevity of its cells, i.e. there is an intrinsic cellular program for the regulation of the life-span. (2) There are constitutional factors (FS and two other factors are known at present) which are correlated with the cellular life-span. These factors are also related to constitutional factors (such as brain weight, body weight and metabolic rate) correlated with the longevity of the organism as a whole. (3) The constitutional factors related to the cellular life-span are most likely under a common regulatory control, which is suggested to be manifested in the cell membrane. (4) The chromosome is organized in a hierarchy of helical coils to form successively higher orders of fiber structures. This condensation (coiling) occurs as a differential process at all levels of fiber organization which leads to a beaded or chromomeric pattern of condensed chromatin along the fiber length. over		
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In view of these results and the primary role of gene regulation in ageing and longevity (data reviewed), a model defining these phenomena has been suggested which involves a general interaction between the genome and the cell membrane. Such an interaction may operate in a way that the genome specifies the composition of the cell membrane which in turn specifies the genome expression. Age-related changes in the membrane composition and function and their consequences for intracellular ionic strength lead to conformational changes in the chromatin structure which ultimately result in repression of genetic activity. The chromosome model presented provides a program for specifying the genetic expression imposed by the cell membrane. Changes in longevity may thus result from structural alterations in the chromosomes by means of "position effects", which effect the differential condensation process regulating gene activity.

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- V. Röhme, D. (1981). Ageing and the fusion sensitivity (FS) potential of human cells in culture: Relation to tissue origin, donor age, and in vitro culture level and condition. - Mech. Ageing Develop. (In press.)
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CELL AGEING AND LONGEVITY: TOWARDS A MODEL BASED ON
EXPERIMENTAL STUDIES IN MAMMALIAN CELL CULTURES

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CONTENTS

I.	INTRODUCTION	7
II.	EVOLUTIONARY AND GENETIC ASPECTS OF LONGEVITY	9
	1. Life-spans	9
	2. Constitutional Factors	10
	3. Gene Regulation or Structural Gene Alterations	11
III.	ORGANISMIC VERSUS CELL AGEING	14
	1. Cellular Life-spans in Culture	15
	2. Constitutional Factors in Cellular Life-span	19
IV.	THE GENETIC APPARATUS	24
	1. Chromosome Model	24
	2. Age-related Changes in the Chromosome	26
	a. Structural Conformation	26
	b. The Primary Structure (DNA)	27
V.	TOWARDS A MODEL	31
VI.	ACKNOWLEDGEMENTS	35
VII.	REFERENCES	36

I. INTRODUCTION

Although life, ageing and death are generally accepted as natural and essential to the evolutionary process, little is known about the factors controlling the life-span and ageing of a species. Basically there are two striking and universal characteristics of all multicellular organisms : (1) they undergo a gradual decline in their adaptability to the normal environment after attaining reproductive maturity and (2) all members of a species have a more or less fixed life-span.

In contrast to multicellular organisms, many unicellular eukaryotes like *Paramecium*, *Amoeba* and *Neurospora* seem to exhibit a finite life-span only under sub-optimal conditions of proliferation except for certain mutants (for review see Smith 1978). One major difference between uni- and multicellular organisms which may be of relevance, is the lack of cell differentiation (cell specialization) in case of the former ones (Pontén 1977).

Like most biological phenomena, there is ample evidence to support both genetic and environmental approaches in studies on ageing (Comfort 1974). As a consequence, numerous theories have been proposed. These theories may implicate programmed ageing, somatic mutation, accumulation of errors, effects of free radicals and/or crosslinking, autoimmunization, neuro-endocrinological control and many others (for review see Rockstein 1974, Shock 1977, Kanungo 1980).

It may be wise to perceive at the start, not only that the topic is elusive, but also that it is posed inside an arbitrary conceptual framework. Superficially, it may appear a relatively simple problem to explore, but the more it is tried to crystalize it with scientific investigation in mind, the more it becomes eroded by elimination of aspects that prove to be irrelevant.

This investigation focuses on comparative analysis of mammalian longevity and the constitutional factors on which this may rely. In particular, emphasis has been put on (1) cellular life-span in culture, (2) constitutional factors related to cellular life-span (especially the cell fusion sensitivity (FS) potential) and (3) the organization of the chromosome.

II. EVOLUTIONARY AND GENETIC ASPECTS OF LONGEVITY

1. Life-spans

Although the life-span of a species is more or less fixed, it has no obvious correlation with phylogenetic level or evolutionary position (Hildeman 1978). Animal species on the low phylogenetic scale of physiologic complexity such as coelenterates may survive just as long (e.g. 70-80 years) as progressively higher bivalve mollusks, turtles, large birds, and mammals (Comfors 1965). However, this does not mean that most species in a given taxonomic class or phylum are similarly long-lived. On the contrary, as may be exemplified by the mammals, the longevity may vary very much, over a 50-fold range (Cutler 1975). Even within the same order of mammals, the longevity may vary 8 to 12-fold as in the case of primates (King and Wilson 1975) and rodents (Biological Data Book 1972). A tabulation of recorded data on the longevity of about 325 animal species, 125 of which are mammals, is presented in Biological Data Book (1972). Although the majority of these data on life-span is subject to considerable uncertainties, the assumption has been made that there is no systematic bias.

Usually estimates on longevity are based on the maximal life-span (MLS) rather than the average one. This is due to the fact that the cause of death in natural populations usually is more related to predation than to senescence (Sacher 1978). The survival of wild populations of small mammals usually can be fitted to an exponential distribution, whereas the same species in captivity under good conditions usually live much

longer and conform to a negatively skewed sigmoid function, the Gompertz distribution (see Sacher 1978), which also characterizes human populations. The major difference between these distributions is that whereas the mortality rate in the former case is more or less age-independent, it is age-dependent in the latter one. In intermediate situations, the age-independent and age-dependent mortality may be of comparable importance, which is accounted for by the composite function known as the Gompertz-Makham distribution. The mathematical basis of these distributions and their fundamental importance to explain evolutionary change in survival characteristics is discussed in detail by Sacher (1977, 1978).

2. Constitutional Factors

Investigation of constitutional factors in mammalian longevity goes back to the beginning of this century (Rubner and Friedenthal, see Sacher 1959) and has more recently been taken up and successfully extended by Sacher (Sacher 1959, 1975, 1978).

About 20 years ago it was demonstrated (Sacher 1959) that the MLS of mammals was related to two extensive variables of body dimensions, the brain weight (E) and the body weight (S). The multiple regression of $\log_{10} E$ and $\log_{10} S$ on $\log$ MLS resulted in the following equation:

$$\log_{10} \text{MLS} = 0.64 \cdot \log_{10} E - 0.23 \cdot \log_{10} S + 1.04 \dots\dots(1)$$

In a subsequent study, this relation was confirmed in an analysis of not less than 239 species of mammals (Sacher 1975), which also established that it was not significantly different within various orders, namely Primates, Rodentia and Artiodactyla. Equivalent relations of MLS to brain weight and body weight also hold true for birds and lower vertebrate classes (Mallouk 1975).

When introducing two intensive variables, the metabolic rate (M) and the deep body temperature (T_b) in addition to brain weight and body weight the multiple regression was given by the equation:

$$\log_{10} \text{MLS} = 0.62 \log_{10} E - 0.41 \log_{10} S - 0.52 \log_{10} M + 0.026 T_b + 0.89 \dots\dots\dots(2)$$

This equation accounts for almost all longevity variance of mammals (Sacher 1978).

3. Gene Regulation or Structural Gene Alterations.

Based on fossil records the rate of evolution of MLS has been determined in certain evolutionary lineages of primates (Cutler 1975, Sacher 1975), ungulates and carnivores (Jerison 1973, Cutler 1976, 1979). These studies revealed periods in which there were dramatic increases in the rates of evolution of longevities. The rapidity of this evolution has lead to the proposition that gene regulation may be of prime importance in determining longevity (Cutler 1975, 1979, Sacher 1976, Sacher and Hart 1978, Martin 1979).

Recent findings of evolutionary biologists (Wilson et al 1977, King and Wilson 1975) indicate that structural gene evolution and morphological evolution or speciation can proceed at independent rates. In mammals, speciation has been occurring much too fast to be accounted for by amino acid substitutions resulting from structural gene mutations. The human and chimpanzee differ very little in macromolecular sequences (DNA and protein) (King and Wilson 1975) but differ greatly in organoid changes (Wilson et al 1977) as well as in longevity. Speciation (Wilson et al 1977, Bengtson 1980) as well as longevity variance (Martin 1979) within different orders of mammals appear to correlate with the extent of chromosomal rearrangements.

Although environmental factors contribute the most to the variation of longevity found within species (see Sacher 1977, Ross 1976), there are also many types of observations that emphasize the influence of genetic factors. In many mammals the offspring of long-lived parents have a longer than average life-span (Comfort 1958, Abbott et al 1974). Monozygotic twins have a similar life-span more often than dizygotic twins (see Bank and Jarvik 1978). In most mammals, females usually outlive males (see Rockstein et al 1977). Moreover, extrachromosomal or cytoplasmic inheritance may play a role in mammalian longevity, since the maternal contribution in case of humans is perhaps twice that of the paternal (Abbott et al 1974).

In an effort to identify genes of significance to the pathobiology of ageing, about 7% appeared to have such a potential effect (Martin 1978, 1979). Using an upper limit of 100.000 loci in man, this would give about 7000 loci of relevance to ageing. However, as pointed out by Martin, this figure probably is much too high due to overlapping of the loci involved in various phenotypes. In any event, the number of loci involved would be at least in the hundreds. The figure, 250 loci, has been suggested by Cutler (1975).

The study of progeroid syndromes of man has demonstrated other important aspects of the genetics of ageing (Martin 1978, Goldstein 1978). Although these syndromes may not represent natural ageing in all aspects, still they demonstrate a number of the major phenotypical expressions of ageing. Interestingly, apart from the rare Werner's and Cockayne's syndromes and Progeria (the three "classical" premature ageing syndromes), the three common chromosomal aneuploidies, Down's, Klinefelter's and Turner's syndromes, were among the syndromes demonstrating most concordances with the phenotypical criteria of ageing (Martin 1979). In fact, Down's syndrome ranked at the top of the list. Since these latter syndromes exclusively represent variations in gene doses, these studies point to the importance of gene regulation in relation to ageing rather than to alterations in structural genes (Martin 1979).

III. ORGANISMIC VERSUS CELL AGEING.

The fundamental cause of death is the body's increased vulnerability with age. The documentation of changes in structural physiological and biochemical characters associated with age is enormous (see Finch and Hayflick 1977, Bergsma and Harrison 1978, Schneider 1978, Kanungo 1980). In man, loss of functional capacity is about 0.8% per year after the age of 30 (Strehler and Mildvan 1960). What is the cause of this natural decline?

Unfortunately, studies of the effects of ageing alone are not likely to tell us much about its causative nature, unless we first know more specifically what to look at. Having in mind the remarkable relations found between longevity and constitutional factors discussed in the previous section, I think investigations along the line of comparative biology may be helpful to gain such information. Moreover, this approach also is satisfying since it relies on the undisputable facts that the life-span of various species, like man and mouse, is distinctly different and that the relations found between longevity and constitutional factors are unbiased (Murphy 1978).

A key question is whether the major determinant for the longevity of a species is regulated at the level of the organism as a whole or has a manifestation in each individual cell. This is not to ask whether an ageing organism consists of ageing cells, which obviously it does. Neither is it to ques-

tion whether the death of the organism is under organismic control in the sense that the decrements with age occurring in some few cell types are more fatal to the organism than are those of others. For the sake of clarifying the issue, the problem at stake perhaps could be formulated more bluntly: is it theoretically possible to make a man out of mouse cells provided we could impose on them the human scheme of organismal regulation? Whatever way we may formulate the problem, it is central to investigate if there is a relation between organismic and cellular life-spans. If such a relation really exists, we have a solid basis for the relevance of studies of constitutional factors at the cellular level.

In principal there are two ways in which this question has been put to test: (1) growing of cells in cultures outside the body, (2) serial transplantation of cells in isogenic laboratory animals. (For a comprehensive review of this latter issue, see Daniel 1977).

1. Cellular Life-spans in Culture

Whether or not normal cells in culture have a life-span related to the longevity of the species has been a matter of great controversy for a long time. Although the hypothesis has been put forward that there is such a relationship (Hayflick 1974), the data to support it has been hard to find.

There are two major problems associated with this kind of a comparative approach. First, variation in technical procedures for establishing cultures in vitro, as well as in the cultivation conditions employed, may effect the results

(see paper I). Secondly and even more troublesome is the tendency of many non-human cultures to be spontaneously transformed into continuous cell lines (Hayflick 1977, Martin 1977, Pontén 1971). In fact, there are only two comparative studies on this matter comprising a relatively reasonable number of species (Stanley et al 1975, paper I). Whereas Stanley et al could not find any relation between the in vitro life-span of normal cells and species longevity, my results clearly favour the existence of such a relationship. Moreover, on average both the life-span of normal fibroblasts in culture and that of circulating erythrocytes has been demonstrated to be proportional to the species longevity by the same function, the square-root of the species' MLS (paper I). These results may indicate the prevalence of a common functional basis regulating the life-span of fibroblasts and erythrocytes and thus operating in replicative as well as post-mitotic cells in vitro and in vivo.

Here I will only briefly present some new data obtained in Syrian Hamster cultures (the detailed data will be published elsewhere). Not only because these data support the previous hypothesis, but also because they demonstrate some interesting new features of relevance to this area.

Out of 16 embryonal fibroblast cultures of Syrian Hamster (whole embryos), 15 demonstrated the replicative life-spans presented in Fig. 1; the 16:th culture had undergone 36 population doublings (PD) and was still proliferating when writing this paper. The variation in life-span among the 15 cultures, 7 to 25 PD's is far beyond that found in other species studied (paper I). Another

striking feature is that the distribution of life-spans seems to be bimodal. However, analysis of the growth kinetics in 10 cultures (including the one still proliferating) revealed the occurrence of a major growth crisis (see paper I) 10-16 PD's prior to the replicative end of the cultures (Fig. 2). The growth kinetic plot shown in this figure represents a culture showing a first major growth crisis after 9 PD's then the culture resumed its growth for another 15 PD's before it entered a new growth crisis and was finally lost. In the remaining 6 cultures there was only one growth crisis which represented the replicative end of these cultures. The distribution of the population doubling levels (PDL) at which the first or the only growth crisis occurred in the 16 cultures is presented in Fig. 3. In contrast to the distribution shown in Fig. 1, the distribution in Fig. 3 demonstrates a more narrow variation and has a clear unimodal appearance. On the average, the first or only major growth crisis occurred after 10 PD's. Preliminary chromosome studies (on orcein stained preparations) in two cultures revealed an apparently normal diploid chromosome constitution in the cell populations obtained after the first growth crisis.

As far as the growth kinetics are concerned, the case with the Syrian Hamster cultures is similar to that of the horse previously reported (paper I). In case of the horse, though, the cells proliferating after the first major growth crisis were characterized by a hypertriploid chromosome constitution. It should be noted that the first major growth crisis in Syrian Hamster occurred around the culture level expected to repre-

sent the proliferative end of normal cells from mammals with this longevity (paper I). We therefore have to consider the possibility that the cell populations after the first growth crisis may be in some kind of altered condition. The nature of such an alteration can only be speculated upon. The analogy to the limited growth potentials of benign tumors in vivo (Pontén 1977) is appealing, though.

It should be noted that Syrian Hamster embryo cells, like those used here, have been reported to senesce in vitro at various passages, 6-25 (Barret and Ts'o 1978, Little 1976). According to Barret and Ts'o, the reason for this variation was attributed to different cultivation conditions. Yet they pointed out that these cultures could be classified in two groups, either having a relatively short or a long life-span. In our case the cultivation conditions were in every respect the same for different cultures. For a comprehensive review of cellular life-spans in vitro, see Pontén (1971), Martin (1977) and Norwood (1978).

In Fig. 4, the data on the incidence of proliferative changes of cell cultures leading to the development of continuous cell lines or the type of changes found in case of Syrian Hamster and horse are presented for the nine species studied. Without going further into details here, it should be pointed out that there seems to be an inverse relationship between the incidence of such proliferative alterations in cell cultures and the life-span of the cells in which they occur. These data are in accordance with a recent review of this matter (Macieiera-Coelho et al 1977).

2. Constitutional Factors in Cellular Life-span

Only very recently it was demonstrated that there are constitutional factors in longevity expressed at the level of cells in culture. The first evidence for the existence of such a factor was given by the work of Hart and Setlow (1974) on the capacity of cultured mammalian fibroblasts to carry out excision repair of thymidine dimers produced in DNA by ultraviolet (UV) irradiation. In a series of 7 species, ranging from the shrew and mouse to elephant and man, a striking direct relationship was found between the initial rate and extent of UV-induced excision repair and the species longevity (MLS).

A second such factor was given by the work of Schwartz and Moore (Schwartz 1975, Schwartz and Moore 1977, 1979, Moore and Schwartz 1978) on the capacity of the same type of cells (cultured mammalian fibroblasts) to convert potential mutagens like 7,12- dimethylbenz(a)anthracene (DMBA) and benzo(a)pyrene (BP) into proximal mutagenic substances. In a series of 6 (8) species, ranging from rat and Guinea pig to elephant and man, a very good inverse relationship was found to the species MLS.

The third such factor presently known (paper II), relates to the capacity of the same type of cells to fuse after treatment with inactivated Sendai virus. In a series of 19 species, covering 10 mammalian orders and species in the whole range of mammalian MLS, also a striking direct relationship was found between the initial rate of cell fusion (fusion sensitivity (FS) potential, paper III) and species MLS.

Is there any basis for a common regulation of these factors? In an effort to gain such information the data reported on the different constitutional factors were calculated on a log/log basis using the same estimates on species MLS. (Table 1). Evidently, the relations of the FS potential and the excision repair with the fibroblast life-span, were the same i.e. these two factors show a direct linear relation to the life-span of the cells in which they are expressed. In case of the DMBA conversion, an inverse relation to the square of the cellular life-span is suggested. This could be a reflection of a cellular mechanism involving two oxidations of this molecule to form reactive metabolite(s), as has recently been suggested from kinetic studies of the conversion of benzo(a)pyrene (see Diamond and Baird 1977).

Hart and Setlow (1974) suggested that the UV-induced repair was related to the logarithm of the species MLS, i.e. this factor should be related to the MLS by an exponential function. Therefore, all other data were also calculated on this linear/log basis, (data not shown), including the relation of fibroblast life-span in vitro and erythrocyte life-span in vivo to the species⁻MLS (paper I). In all cases, the correlation was less strong than those presented in Table 1, except for UV-induced repair which was about the same.

As pointed out by Martin (1977) and Norwood (1978), there is the possibility that these apparent constitutional factors in cellular life-span may in fact rather be an expression of functional heterogeneity at the cell population level. However,

in case of the FS potential, this possibility can be ruled out, since each cell in the population seem to participate in the fusion process with the same probability (paper IV). It is also noteworthy that the FS potential and extent of UV-induced excision repair demonstrate concomitant changes in human cells grown under different serum conditions (Table 2). When the serum was changed from FBS1 to NCS3 (or the vice versa, Röhme in preparation), a 35-40% decrease was observed to be manifested in both parameters within 24 hours. This is not likely to result from any significant change in the cell population structure, but rather it strongly indicates that there is at least one intracellular level of common regulation for these factors. Thus, most likely the parameters discussed here represent expression at the level of individual cells, i.e. are constitutional factors in the cellular life-span.

Even though it is obvious that the relations found for the various constitutional factors and the cellular life-span are merely suggestive and have to be established in larger comparative studies, the striking similarities indicated, urge the question whether there may be any functional relationship between these factors. As far as effector mechanisms are concerned, it certainly is hard to conceive how the repair of DNA may be functionally related to the metabolism of hydrocarbons or functions of the cell membrane (as probed by the FS potential, paper III). Thus, if there is any functional relation, most likely this has to be related to the regulation of the efficiency of these factors.

As indicated from the experiment presented in Table 2, at least one such level of common regulation may be related to the function of the cell membrane. A relation between the function of the cell membrane and the efficiency of UV-induced excision repair also has been suggested by other investigators (Milo and Hart 1976). A role of the membrane composition (at least intracellular membranes) as an initial rate limiting step in the metabolism of hydrocarbons is established (see Nebert and Atlas 1978).

That the cell membrane may be a constitutional factor for the regulation of cellular life-span is consistent with a number of reports of various cell membrane alterations observed in ageing fibroblasts (see paper V) and erythrocytes (see Marikovsky et al 1977). Indeed, in case of the erythrocyte, the function of the cell membrane no doubt is the key factor in the regulation of its life-span (Kay 1975, Marikovsky et al 1977). The fact that the life-span of cultured fibroblasts and the FS potential of these cells appear to be related to the species life-span in the same way as is the life-span of erythrocytes in vivo (paper I), may be taken as an indication for a similar key role of the cell membrane in fibroblast life-span.

Without going into details here, it may be worthwhile to point out the importance of the cell metabolism in relation to fibroblast life-span in culture as implicated by the effect of temperature (Thompson and Holliday 1973) and oxygen (Packer and Fuehr 1977) during cultivation. It should also be noted that there is the possibility that the constitutional factors

in cellular life-span may be simply related to the constitutional factors of longevity at the level of the organism as suggested from the equations given in Section II:2 and Table 1. For example the metabolic rate of the organism could be related to the inverse forth power of the FS potential.

Lastly, if the cell membrane has a key role in regulating the life-span, what is the constitutional role of the UV-induced excision repair and the polyhydrocarbon metabolism in cellular life-span? Superficially the longevity relation of these factors could be interpreted as ideal cases to support the "intrinsic mutagenesis" theory of ageing (Burnet 1974). According to this theory, the cellular life-span would be regulated by the rate of induction of somatic mutations. However, in case of UV-induced excision repair this is not likely, since cultured fibroblasts from patients with Xeroderma Pigmentosum (XP) which have defective excision repair, do not show premature senescence (Goldstein 1971). Other evidence against a primary role of somatic mutations in relation to ageing will be discussed in section IV:2. Instead, the increased incidence of cancer in XP patients (see Kraemer 1977), rather suggest a role for excision repair in relation to cancer. Such a role also may be true for the efficiency of the polyhydrocarbon metabolism (see Kellerman et al 1978). Thus, there is the possibility that cellular mechanisms for protection against disease caused by effects on the chromosome are not per se involved as effector mechanisms in the ageing process, but merely share a common regulation with the primary mechanism(s) for this process.

IV. THE GENETIC APPARATUS

1. Chromosome Model

The chromosomes of higher organisms consist of the genetic material, DNA, complexed with essentially two major classes of proteins (histones and non-histones) and RNA. The organization of the chromosome, both the structural conformation and the base sequence of DNA are critical for its proper function. Although functionally an integrated structure as a whole, the molecular complexity of the chromosome and its very large size, has made it necessary to study its organization at different levels with different approaches and employing a vast number of techniques. Thus, the research on chromosome structure has become a highly multi-disciplinary field. A comprehensive current review of major aspects in this field as a whole is given in Cold Spring Harbor Symposia on Quantitative Biology (1978); for other references see paper VIII.

A prominent feature of the structural organization of the chromosome is the occurrence of a variety of chromosome fibers which represent different degrees of DNA compaction (see paper VIII). What are the mechanisms for the condensation process at the various levels of fiber organization? How are the fibers organized in chromosome structural units such as the chromosome? These are major questions dealt with in various ways in different chromosome models (for ref. see paper VIII). Furthermore, any such model has to be consistent with the basic concept of differential gene activation.

Our studies in this field has been focused on premature chromosome condensation, PCC (papers VI, VII, VIII). In particular, the technique with PCC has proved to be a valuable tool in unravelling the chromosome structure at levels intermediate to its lowest and highest order of organization, which is presently not easily accessible by other techniques. Based on these studies, a chromosome model has been suggested comprising a hierarchy of helical coils to form successively higher orders of fiber structures (paper VIII). Central to this model is also that the condensation occurs as a differential process at all levels of fiber organization leading to the prevalence of a beaded or chromomeric pattern of condensed chromatin along the fiber length. The possible implication of this chromosome model in relation to regulation of genetic activity is discussed in paper VIII.

Lastly, emphasis should be made on the crucial role of different ions like K^+ , Na^+ , Ca^{2+} and Mg^{2+} in regulating the conformational status of the chromosome (see Lezzi and Roberts 1972, MacLean and Hilder 1977, Sedat and Manuelidis 1978, Ris and Korenberg 1979, Marsden and Laemmli 1979). Evidence that the changes in the concentration of these ions is related to decondensation or condensation of specific chromomeres and changes in their genetic activity has been elegantly demonstrated in *Chironomus* polytene chromosomes (see Lezzi and Roberts 1972). It is noteworthy that according to the model of differential condensation discussed (paper VIII), changes in ionic concentration would lead to specific changes in the pattern

of condensation or decondensation and thus relate specifically to the regulation of genetic activity.

2. Age-related Changes in the Chromosome

a. Structural conformation

Many properties related to the structural conformation of the chromosome have been observed to change with age. Most prominent is the demonstration of the increased melting temperature (see Cutler 1978). Consistent with this is the finding that the amount of protein that can be salt-extracted from chromatin also decreases with age (O'Meara and Herrmann 1972, Müller et al 1975). These observations may indicate a stronger binding of proteins to DNA with increasing age, probably involving covalent crosslinks between proteins and DNA (Hahn 1970).

Whereas the total content of histones appear to remain fairly constant throughout the life-span, the three subfractions of histone H1 (the modifications induced by phosphorylation, acetylation and methylation) may undergo specific changes at least in certain tissues (Medvedev et al 1977, 1978). The data on age-related changes in non-histone proteins are inconsistent (Kurtz and Sinex 1967, Pytilä and Sherman 1968, Berdyshev and Zhe-laboskaya 1972, Dell'Orco et al 1978). However, analysis of specific types of nuclear non-histone proteins indicate an age-related increase (see Hahn 1970, Dell'Orco et al 1978). Furthermore, the chromatin has been demonstrated on a morphological basis to be more condensed in old cells, both in post-mitotic (Zs. Nagy and Zs. Nagy 1975) and replicative ones (Johnson Jr 1979)

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EVIDENCE FOR A RELATION BETWEEN LONGEVITY OF MAMMALIAN
SPECIES AND LIFE-SPANS OF NORMAL FIBROBLASTS IN VITRO
AND ERYTHROCYTES IN VIVO

(cell ageing/replicative potential/cell cultures/growth crisis/
chromosomes)

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Abbreviations: PD, population doubling; PDL, population doubling
level; MLS, maximal life-span

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SUMMARY

The replicative life-spans of mammalian fibroblasts in vitro were studied in a number of cell cultures representing eight species. Emphasis was put on determining the population doubling level at which growth crisis and/or chromosomal alterations occur. All the cell cultures studied went through a growth crisis, a period of apparent growth cessation lasting for at least two weeks. In most cultures, the crisis represented the end of their replicative capacities, but in some cultures cell proliferation was resumed after the crisis. A predominantly diploid chromosome constitution (more than 75%) was demonstrated prior to the growth crisis. In cultures where cell proliferation was resumed after the crisis, a non-diploid constitution was prevailing in all cases except the rat (with 90% or more diploid cells all the time). The growth crisis occurred at PDL's that were characteristic for the species and was shown to be related to the species' maximal life-span (MLS) by a strict power law relation, being proportional to the square-root of the MLS. Based on recorded data (Biol. Data Book 1972), the same relation was also valid for the life-spans of circulating mammalian erythrocytes in vivo. These results may indicate the prevalence of a common functional basis regulating the life-span of fibroblasts and erythrocytes and thus operating in replicative as well as post-mitotic cells in vitro and in vivo.

INTRODUCTION

A hypothesis has been put forward by Hayflick that there is a direct relationship between the species longevity and the replicative potential of its normal fibroblasts in vitro (1). This is mainly based on the recorded data in the literature on the three most studied species in this respect, the two mammals, man and mouse, and the chicken. The primary purpose of this investigation is to verify whether or not this holds true when extending the number of mammalian species studied. According to Stanley et al (2) it does not, but their results are not well-founded.

That normal human fibroblasts in culture have a limited replicative life-span is well established (3-6). In the case of cultures derived from human embryos, which are frequently used for studies on in vitro life-spans, cell proliferation is usually sustained for 40 to 70 population doublings (PD) (4-6). Since there is an inverse relation between the replicative potential of human fibroblast cultures and donor, this cellular system has been suggested as a possible model for ageing in vitro (7-10).

Although less well studied, a limited replicative life-span also seems to characterize normal fibroblastic cultures from animal species (4,11). However, a comparative approach to assess the replicative life-spans of normal animal cells is complicated by the tendency of many non-human cultures to be spontaneously transformed into continuous cell lines (4,11,12). The chromosomal constitution is certainly most significant in this connection, since most continuous cell lines are not normally diploid. However, there are a few controversial cases, including the rat (13,14) and the fat-tailed dunnart (2,15) fibroblast cell lines, and some human lymphoblastoid cell lines (16). It should be noted though that Epstein-Barr virus transformation of lymphoblastoid cell lines is accompanied by

chromosomal abnormalities, although there is a long lag time between the initiation of the transformation and the induction of these abnormalities (17). Most likely chromosome abnormalities eventually occur in all continuous cell lines (4,11,12).

Central to the original concept of the limited in vitro life-span of human cells was the demonstration of phase III, a period of apparent decrease in the rate of cell proliferation accompanied by morphological and cytological changes, which leads to the proliferative end of the culture (3,7). In animal cell cultures like mouse, rat and rabbit, a growth crisis, which is similar to that of phase III, has been demonstrated at an early level of culture in many continuous cell lines (13,14,18-21). The growth crisis may be an indicator of the replicative end of animal cells and could thus be an expression of phase III, since in case of mouse and rabbit most of the cells after the crisis had changed chromosomal constitutions (18,19,21).

In the present study, quantitative assessments of growth rates and chromosomal constitutions have been made in serial passages of a number of fibroblastic cell cultures from eight mammalian species. The results support the concept of a direct relationship between cellular life-span and species longevity (1).

MATERIALS AND METHODS

Biopsies. Biopsies were obtained from the lung, skin, testis or the whole embryo of one or more male individuals belonging to eight mammalian species (Table 1). In all cases except for horses, the biopsies were taken after the death of the individuals. Horse biopsies were obtained in connection with castration from the testicular capsule (Tunica albuginea) and from the skin at the incision of the castration operation. The skin biopsies were principally composed of the superficial dermis. In the case of the mouse (Swiss strain), rat, and man, all material was taken from embryos, whereas the biopsies from the rat-kangaroo, mink, rabbit, bat and horse were all from adult animals. Except for the horses which were all 2-4 years of age, the ages of the adult animals are not known.

Cell cultivation. The biopsies were immediately transferred to test tubes with 5 ml tissue culture medium. - Eagle's basal medium with Hanks' salts supplemented with 15% fetal bovine serum (BIO-CULT), 100 µg/ml streptomycin and 100 IU penicillin, pH=7.2. After washing in fresh medium, the biopsies were minced with scissors and plated in 2-4 ml dilution bottles. The time needed to achieve confluent layers of fibroblastic cells varied with the tissues, individuals and species. Confluent cells were detached from the glass by first rinsing them quickly with isotonic sodium citrate solution before treatment with 0.25% trypsin (Difco) in Puck's saline A. After suspension in complete medium, the cells were transferred to new bottles. Routine subcultivations were carried out once or twice a week at split ratios 1:2, 1:3 or 1:4 depending on the rate of growth. This procedure ensured that the cells were predominantly in the exponential phase of growth during the entire cultivation period.

The number of PD's was calculated from the split ratios (S) by the formula $PD = \log (1/S) / \log 2$ (as modified from ref. 22), which for the split ratios of 1:2, 1:3 and 1:4 gives 1, 1.6 and 2 PD, respectively. The culture age was calculated as the PD level (PDL) starting from the first in vitro passage.

The cultures were carried in at least one of the two main batches of fetal bovine serum used; a few cultures were also carried in serum from other batches.

After initiation and cultivation to PDL 2-5, all cultures were frozen in complete medium supplemented with 10% dimethylsulfoxide and stored in liquid nitrogen. Freezing to -70°C was done in liquid nitrogen vapor at an estimated rate of $1^{\circ}/\text{minute}$. Growth kinetics were studied in cultures initiated from biopsies or frozen stocks (PDL 2-5). In the latter cases, confluent monolayers were obtained one or two days after the cells have been thawed.

Chromosome preparations. Conventional air-dry preparations were made according to the standard procedure at this laboratory (23).

RESULTS

Growth crisis

The occurrence of a growth crisis was determined from growth kinetics plots as exemplified for mouse and human cultures in Fig. 1. Whereas all the mouse cultures passed through an apparent growth crisis at PDL 8-11 for about 2-3 weeks before cell proliferation was resumed, phase III characteristically represented the end of the replicative potential of the human cultures. In the cases of mouse, rat and bat, transformation into continuous cell lines occurred 2-3 weeks after the crisis in 5 out of 5, 3 out of 4 and 1 out of 3 cultures, respectively. Also in case of the horse, cell

proliferation was resumed after the crisis in 2 out of 12 cultures, but these cases differed from those of the mouse, rat and bat. Not only was the length of the crisis longer in these horse cultures, but also cell proliferation after the crisis was comparatively slow and continued only for about 10 PD before the culture reached its ultimate replicative end (Röhme, unpublished data).

Based on the growth kinetics plots as shown for mouse and man in Fig. 1, the PDL's at the growth crisis for all the cultures are summarized in Table 1. Evidently the major source of the overall variation is related to the species origin of the cultures. Between the extremes, man and mouse, the difference was about 6 to 7-fold. With regard to different individuals, tissues of origin and batches of serum used, the variation is usually $\pm 20\%$ of the species mean, except for the horse cell cultures which was $\pm 40\%$.

Chromosome analysis

Chromosome numbers in 50 cells and the gross morphology of the chromosomes in 10 cells were determined in at least one culture from each species. Table 2 shows that the cells with diploid numbers continuously decrease in frequency during the cultivation period in all cases except the rat. In all cultures except those from the rat and bat, the diploid cells represented some 75 to 85% of the cells just prior to the growth crisis. In the case of the rat, the diploid cells represented some 90% of the cells at this stage of the culture, but increased somewhat in the cell population after the growth crisis. In case of the bat culture, the cell population after the growth crisis was represented by about equal numbers of diploid cells ($2n=38$) and cells with 37 chromosomes. In accordance with previous findings of mouse cultures (18,19), the cell

population obtained after the crisis were dominated by hypotetraploid cells with 71 to 79 chromosomes ($2n=40$). In case of the horse culture, the cell population after the growth crisis was hypertriploid with more than 75% of the cells having 97 to 104 chromosomes ($2n=64$).

Fibroblast and erythrocyte life-spans and species longevity

In Table 3 the PDL's at the growth crisis (given in Table 1) are averaged and listed in the order of increasing maximal life-span (MLS) of the species. The PDL at the growth crisis is nicely related to the species longevity. In fact, calculated on a logarithmic basis ($\log/\log$), the relation between the PDL at the growth crisis and the MLS of the species follow a strict power law relation (Fig. 2). The linear regression coefficient of MLS on PDL is 2.07, and the correlation coefficient, 0.95, is highly significant ($p<0.001$). A point to be considered here is that the human, mouse and rat cultures are of embryonal origin, whereas those of the other species are from adult donors. However, most likely this difference is not crucial to the relation found here. Reduction of the PDL's of the embryonal cultures by a factor of 25% (Table 3), which corresponds to the average difference found in human cultures of embryonal and adult (30-40 years of age) origin (8), does not have much effect on the regression coefficient, which in this case is 1.99. The correlation also is as strong as before, the coefficient being 0.95.

Based on the published data of the in vivo life-span of circulating mammalian erythrocytes (25), a similar comparison with the species MLS is presented in Fig. 3. The MLS is here calculated as a function of the erythrocyte life-span merely to facilitate the comparison with the fibroblast case. Since different techniques

have been employed to determine these life-spans, the calculations are based on the mean of the lowest and highest value recorded for each species. The regression coefficient of the life-span (days) on MLS is 1.96, and the correlation coefficient 0.77, which is clearly significant ($p < 0.01$).

DISCUSSION

A direct relation was found between the longevity of 8 mammalian species and the occurrence of growth crisis in their cultured fibroblasts. This demonstrates that the growth crisis is a representative limit for the in vitro replicative potential of "normal" animal cells and may be equivalent to phase III of human cells (1,3,7). The major implication of this is that this potential is primarily determined by an intrinsic cellular program (3,7).

Unfortunately, direct comparisons of the replicative potentials of normal cells in culture obtained in different studies are hampered by the fact that they may vary considerably due to differences in tissues of origin (11,28) and cell types (29) as well as to the specific culture conditions employed, including the nutrient medium, serum and cultivation procedures (30-34). Also intraspecific genetic differences may play a role as suggested from the fact that fibroblast cultures from humans who age prematurely, Werner's syndrome and progeria, may undergo less PD's if compared to normal cells (8,35,36). However, it should be noted that the timing of the growth crisis and/or chromosomal changes obtained here are within the range of the variation previously reported for the well studied species such as man (4,6), mouse (18-20), rat (13,14) and rabbit (21).

Stanley et al (2) could not find a relation between species longevity and the replicative potential of its normal cells. However it is difficult to interpret this study, since the time of onset of transformation was arbitrarily determined by the time of appearance of heteroploidy without simultaneous quantitative assessments of growth rates in serial passages. Thus, as exemplified by the case of rabbit, Stanley et al reported a normal life-span of 70 PD, whereas Martin and Ogburn (21) observed a growth crisis at about PDL 20, the same level as demonstrated here, after which a pseudoploid cell population resumed cell proliferation showing only subtle chromosomal alterations.

That the life-span of circulating erythrocytes appears to be related to the species longevity in the same way as cultured fibroblasts is remarkable not only because they represent another cell type, but also because they are post-mitotic and concern in vivo conditions. A square-root relation to the species longevity of cellular life-spans as suggested here for erythrocytes and cultured fibroblasts, also may apply to the in vitro survival of lymphocytes as recorded by Ling and Kay (37). Although these data are incomplete, they still indicate that the 25% survival of lymphocytes is 1.9 ± 0.4 days for the four most short-lived species with an average MLS of 4.2 ± 1.1 years, compared to the 6.8 ± 1.9 days for the four most longlived ones with an average MLS of 44.5 ± 22.1 years. The survival ratio between these groups of cultured lymphocytes, 3.6, is about the same as the square-root of the MLS ratio, 3.3, between these groups of species. Thus, despite the present paucity of data with bearing on comparative cellular life-spans, the results discussed here on fibroblasts, erythrocytes and perhaps also lymphocytes are suggestive of the existence of a common functional basis for the regulation of the life-span operating both in vivo and in vitro.

In case of erythrocytes, convincing experiments demonstrate that the mechanism directly involved in the elimination of senescent erythrocytes from the body is apparently the selective attachment of immunoglobulin G (IgG) to the cell surface of old cells, which functions as a marker for phagocytosis of these cells (38). The different life-spans of erythrocytes from various species is thus likely to be dependent on the rate by which the cell membrane or surface is changed to allow such IgG attachment. Thus, if there is a common basis for the limited life-spans of erythrocytes and fibroblasts as suggested by the present results, this would also imply a primary role of the cell membrane or surface in the regulation of the fibroblast life-span.

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Table 1. The population doubling levels (PDL) at the time of the growth crisis in the different cell cultures studied

Species	Cell culture*	PDL [†] at growth crisis				
		Lung	Skin	Testis	Whole embryo	Average
Mouse	ME 1				11 9	10
<u>Mus musculus</u>	ME 2				9	9
	ME 3				8 9	8.5
Rat	RES 1		13			13
<u>Rattus norvegicus</u>	RES 2		16			16
	RES 3		12 10			11
Rat-Kangaroo	PTL 2	13 16				
<u>Potorous tridactylus</u>	PTS 2		10 12			11
Mink	MVI	27 23				25
<u>Mustela vison</u>						
Rabbit	RL	24 21				22.5
<u>Oryctolagus cuniculus</u>						
Bat	VML	18 20 16				18
<u>Vespertilio murines</u>						
Horse	ECT 1			36 33		34.5
<u>Equus caballus</u>	ECT 2			36		36
	ECS 3		30			30
	ECS 4		39			39
	ECT 4			41		41
	ECS 5		17			17
	ECS 6		17			17
	ECS 8		23			23
	ECT 8			25		25
	ECT 9			25 23		24
Man	HES 5		59 67			63
<u>Homo sapiens</u>	HEL 2	57 62				59.5

* The numerical designation of the culture refers to the individual of origin.

† All values are given as rounded off integers except in the case of the averages. More than one value per culture represents cultivations in different sera.

Table 2. Frequency of diploid cells during the in vitro cultivation of cells representing the eight species studied.

Species	Cell line	Per cent diploid cells at different PDL †																											
		2	4	6	7	8	9	10	12	13	15	16	18	21	23	25	28	29	31	37	38	39	47	53	55	57	59	62	67
Mouse	ME 2	88	—	76	—	—	—	—	↓	—	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Rat	RES 1	96	—	95	—	—	—	—	—	90	—	—	↓	92	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Rat-Kangaroo	PTS 2	96	—	—	—	88	—	—	—	—	84	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Mink	MVI	—	95	—	—	—	—	—	—	93	—	—	—	93	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Rabbit	RL	95	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Bat	VM	96	—	—	—	—	—	—	—	96	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Horse	ECS 4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Man	HES 5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
"	"	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
HEL 2	HEL 2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
"	"	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

† Arrows indicate the occurrence of growth crisis and lines the period of growth of the culture.

Table 3. The average population doubling level (PDL) at the growth crisis and the maximal life-spans (MLS) of the eight species studied

Species	PDL at growth crisis			Number of cultures	Species MLS (years)
	Range	Mean	$\pm$ SE		
Mouse	8 - 11	9.2	$\pm$ 0.5 (6.9)*	5	2 (24)
Rat	10 - 16	12.8	$\pm$ 1.3 (9.6)	4	3.5 (25)
Rat-kangaroo	10 - 16	12.8	$\pm$ 1.3	4	7 (2)
Mink	23 - 27	25.0	$\pm$ 2.0	2	10 (25)
Rabbit	21 - 24	22.5	$\pm$ 1.5	2	13 (25)
Bat	16 - 20	18.0	$\pm$ 1.2	3	13.8†
Horse	17 - 41	28.8	$\pm$ 2.4	12	46 (25)
Man	57 - 67	61.3	$\pm$ 2.2 (46.0)	4	110 (27)

* In brackets, the means of embryonal cultures are reduced by 25%.

† Based on the average MLS for species in the subfamily Vespertilionae to which the bat belongs (26).

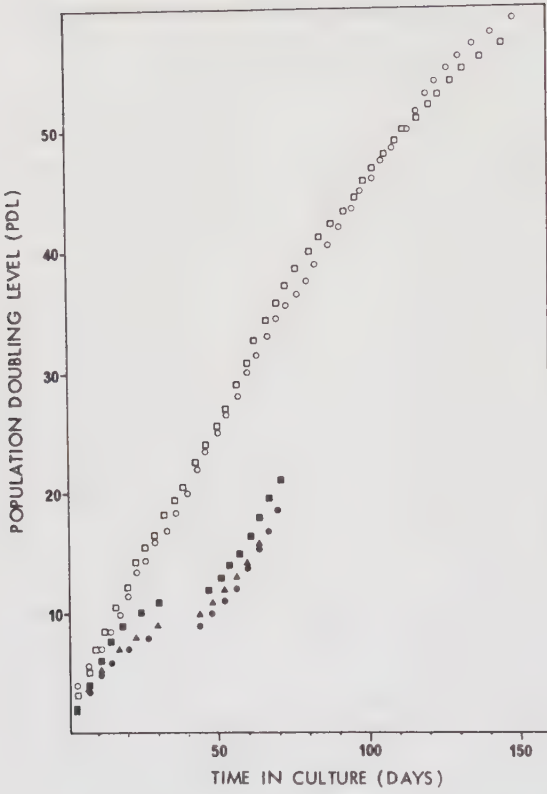


Fig. 1. Growth curves of two human (light symbols) and three mouse (dark symbols) cultures of embryonal fibroblasts.

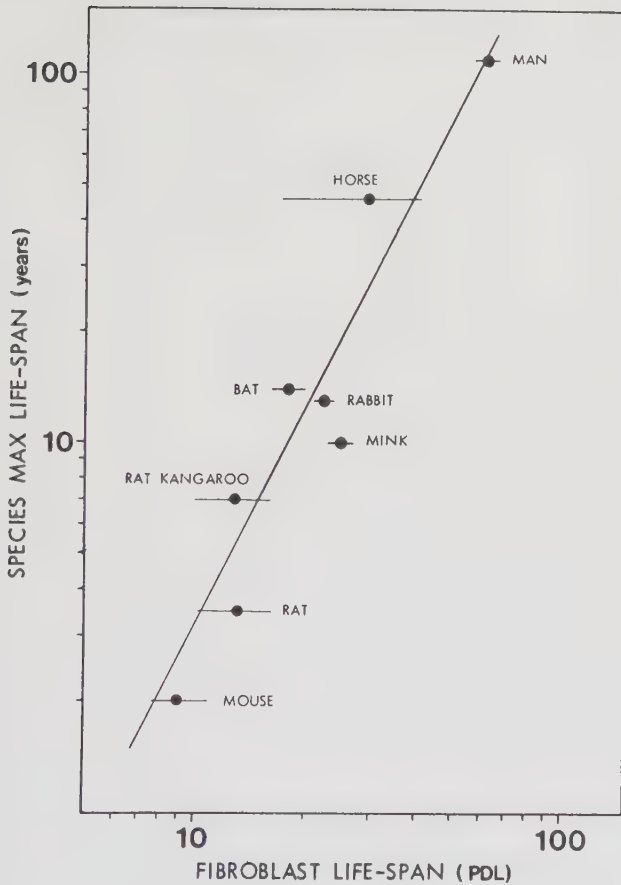


Fig. 2. Linear correlation (log/log) between species maximal life-span, MLS (years), and their fibroblast life-spans in vitro (PDL) based on the data given in Table 3. The linear regression of $\log_{10} \text{MLS}$ on $\log_{10} \text{PDL}$ is given by the equation $\log_{10} \text{MLS} = 2.07 \log_{10} \text{PDL} - 1.58$. The correlation coefficient is 0.95.

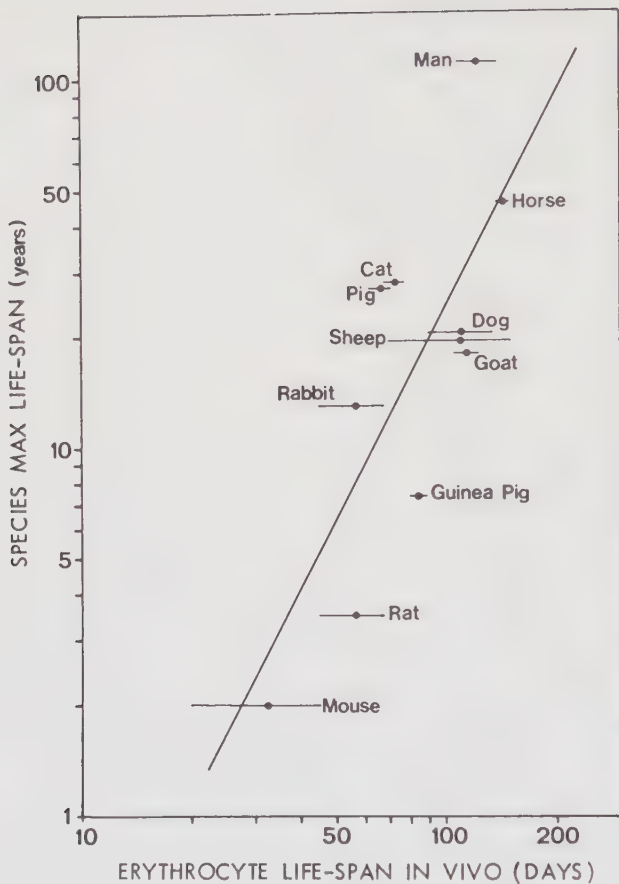


Fig. 3. Linear correlation (log/log) between species maximal life-span, MLS (years), and their erythrocyte life-span in vivo, T (days), based on recorded data (25). The linear regression of $\log_{10} \text{MLS}$ on $\log_{10} T$ is given by the equation $\log_{10} \text{MLS} = 1.96 \log_{10} T - 2.54$. The correlation coefficient is 0.77.

CORRELATION OF THE CELL FUSION SENSITIVITY OF CULTURED
MAMMALIAN FIBROBLASTS WITH THE SPECIES' LIFE-SPAN

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Sendai virus has the ability to induce cell fusion in probably all cultured vertebrate cells, although at a markedly different rate¹⁻⁴. Except for the differences related to the culture conditions employed^{5,6}, the fusion sensitivity (FS) of cells seems to be related to the tissue of origin^{1,5} and the replicative potential of the culture in vitro^{1,7,8}. In particular, FS appears to be higher in cancer cells and continuous cell lines compared to diploid finite cell lines. Sporadic data in the literature also indicate that the species' origin of cells may play an important role in determining the FS^{1,3,7,8}. The fact that the potency of Sendai is higher in human compared to rodent cells⁸, may in an evolutionary context perhaps not be surprising since Sendai is a primate virus¹¹. However, Röhme⁸ also pointed out the possibility that the FS may be related to the species' life-span. Since the species' life-span largely varies independently of phylogenetic or evolutionary position¹², FS studies in a fairly large number of species should be able to discriminate between these two possibilities. The present study shows that the FS of normal mammalian cells in culture is related to the species' life-span.

The FS of low-passage skin or lung fibroblast cultures were determined for 19 mammalian species belonging to 10 orders (Table 1). This collection covers (a) species with extremely different maximal life-spans (MLS), (b) comparatively closely related species (belonging to the same order) with large differences in MLS, (c) comparatively distantly related species (belonging to different orders) with about the same MLS and (d) closely related species (belonging to the same family) with about the same MLS.

The FS comparisons between species were made more reliable by taking into account the expected effect of the donor age (embryonal or adult) of the culture, which has been demonstrated for

human fibroblasts¹⁸. This was done by calculating the species fusion sensitivity (SFS) potential, which is the relative FS value obtained in relation to human embryonal fibroblasts, SFS=1. With regard to the tissue of origin of the fibroblasts, lung or skin, this is not likely to have any major effect on the SFS potential¹⁸.

The most extreme species with regard to MLS, Man (110 years) and shrew (1.5 years), also represented the extreme SFS potentials, 1.0 and 0.15, respectively. This represents a difference in SFS of about seven times. Closely related species on the other hand, belonging to the same family, such as the Harp and the Ribbon seals or the rat and the mouse, are characterized by similar MLS as well as SFS potentials. Distantly related species belonging to different suborders within an order, such as the pig and the Indian muntjac in the order of artiodactyla, the squirrel and the muridae species in the order of rodentia, or the hedgehog and the shrew in the order of insectivora, represent more pronounced differences in MLS as well as SFS potentials.

The orders carnivora and rodentia, which are represented by four species each, have the average SFS values of 0.65 and 0.30, respectively. Compared to the difference in MLS between an average carnivore and rodent, 26 and 6 years, respectively, it thus appears that a two-fold increase in SFS is correlated to a four-fold increase in MLS, i.e. the SFS appears on the average to be proportional to the square-root of the MLS. In fact, such a relation between the SFS potential and the MLS seem to be valid for all the mammalian species studied (Fig. 1). The least squares regression of the logarithm of the MLS in years on the logarithm of SFS is given by the equation

$$\log_{10} \text{MLS} = 2.07 \log_{10} \text{SFS} + 1.81$$

The correlation coefficient being as high as 0.97.

Although cell fusion per se is normally restricted to very few cell types in vivo¹⁹, cell membrane fusions are involved in many basic cellular functions related to the secretory and excretory processes²⁰⁻²³. Thus, there is the possibility that cell fusion may represent an exaggeration of cells to carry out similar normal processes. Alternatively, the life-span relation of the FS potential could be an expression of other cell membrane and/or cytoskeleton function(s). Of significance is that the cell fusion experiments were carried out on trypsinized cells. Apart from removing certain glycoproteins from the cell surface and make others more accessible^{24,25}, trypsinization of cells also influences the function of the cell membrane/cytoskeleton^{26,27}. The importance of this system in relation to the FS potential may be indirectly supported by the lack of correlation between the degree of Sendai virus adsorption and the extent of fusion²⁸, and by the indication of a correspondence in the cellular sensitivity to polyethylene glycol and Sendai mediated cell fusion (Höglund and Röhme, unpublished). The changes effected by the Sendai virus and other fusion agents appear to be related to the enhancement of membrane "fluidity", membrane component mobility, and the creation of particle-free lipid areas²⁹⁻³². Thus, the most likely interpretation is that the cellular manifestation of the FS potential primarily resides in the cell membrane/cytoskeleton structure.

Finally, it is of great interest to note that the replicative life-spans of diploid mammalian fibroblasts like those used here, appear to be correlated to the species longevity with the same function as that demonstrated for the FS potential (Röhme, in preparation).

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Table 1 The species' origin and the SFS potentials of nineteen normal mammalian fibroblastic cultures.

Classification ¹¹	and name of the species	Tissue of origin of cells	Sex	Fetal/adult ^a	Fusion ^b Sensitivity (FS) × 10 ³	Species ^c FS (SFS)	Maximum lifespan (years)
Eutheria							
Artiodactyla							
Ruminantia	<u>Muntiacus muntjak</u> (Indian Muntjac)	skin	Male	adult	6.23	0.44	15 ^d
Suiformes	<u>Sus scrofa</u> (Swine)	lung	"	"	10.88	0.77	27 ¹²
Perissodactyla							
Hippomorpha	<u>Equus caballus</u> (Horse)	skin	female	"	11.96	0.85	46 ¹²
Osteacea							
Mysticeti	<u>Balaenoptera acuterostrala</u> (Lesser Rorqual)	lung	"	"	13.66	0.97	80 ¹²
Carnivora							
Pinnipedia	<u>Histiophoca fasciata</u> (Ribbon seal)	skin	male	"	10.21	0.72	34 ¹²
	<u>Pagophilus groenlandicus</u> (Harp seal)	lung	"	"	10.86	0.77	34 ¹²
	<u>Mustela vison</u> (American mink)	"	"	"	7.19	0.51	10 ¹²
Fissipedia	<u>Felis catus</u> (Domestic cat)	"	"	newborn	11.70	0.66	28 ¹²

Lagomorpha

Oryctolagus cuniculus (European rabbit)" " adult 5.80 0.41 13¹²

Rodentia

Squiromorpha

Sciurus carolinensis (Grey squirrel)

" female

15¹²

Myomorpha

Cricetulus griseus (Chinese hamster)

" male

2.5¹³Rattus norvegicus (White laboratory rat)

" "

3.5¹²Mus musculus (White laboratory mouse)

" "

2¹⁴

Primate

Anthropoidea

Macaca mulatta (Rhesus macaque)

lung

"

29¹³Homo sapiens (man)

lung & skin

"

110¹⁵

Chiroptera

Vespertilio murinus (Particoloured bat)

" "

7.94

13.8¹⁶

Insectivora

Sorex araneus (Common shrew)

" "

2.18

1.5¹²Erinaceus europaeus (European hedgehog)

" "

3.75

4.2¹²Metatheria

Marsipualia

Potorous tridactylus (long-nosed Rat-kangaroo)

" "

3.62

7¹⁷

Notes to Table 1

(a) The age of the adult animals is not known in most of the cases.

(b) Cell fusion was induced in suspensions of trypsinized cells by inactivated Sendai virus according to procedures previously described⁸. Briefly, cell suspensions were obtained from exponentially growing cultures by first rinsing in isotonic sodium citrate followed by treatment with trypsin (0.25% in Puck's solution A). After being aspirated in growth medium (Eagle's basal medium supplemented with 15% fetal bovine serum), the cell suspensions were washed in medium without serum and treated with the virus preparation in test tubes (10^6 cells per 100 μ l of total solution). After 1 hour (15 min at 4°C followed by 45 min at 37°C in a water-shake bath), the cell suspensions were aspirated in growth medium, plated out at a relatively low cell density in petri-dishes containing coverslips and incubated in an atmosphere of 95% air and 5% CO₂ at 37°C. After 17 hours, the coverslips with attached cells were fixed, stained and mounted on slides. The slides were analysed microscopically and the average number of nuclei per cell in the experimental (ANC_E) and the untreated control (ANC_C) cultures were calculated from about 1000 cell analyses. The extent of fusion induced by the virus was calculated as $FI = (ANC_E - ANC_C) / ANC_C$, where FI is the fusion index⁸. The FS potential was determined as the linear rate constant of FI on the virus dose (FAU), i.e. $FS = FI / FAU$, when $FI < 0.1$ ⁸. The FAU (fusion activity units) was determined from the fusion experiments with a reference cell culture, human Lu106, always carried in parallel with the one or two cell cultures to be determined at a time⁸. The FS values given in the table represent the means of two independent experiments of each cell culture, and these values in turn were averages of two replicas.

continuation of the notes to Table 1

- (c) SFS potentials were calculated relative to the FS of human embryonal fibroblasts, $SFS=1.00$. In cases of species where the cultures were derived from adult donors, the FS values obtained were first recalculated by use of the ratio embryonal/adult valid for human fibroblasts, 1.25^{18} . The FS was determined in passage 2.4 in cases of species with MLS of ten years or less, and in passage 5-15 in the other cases.
- (d) This estimate is based on the life-spans of other small deers in Biology Data Book¹².
- (e) This FS value represent the average of seven human embryonal fibroblastic cultures, including four derived from the lung and three from the skin¹⁸.

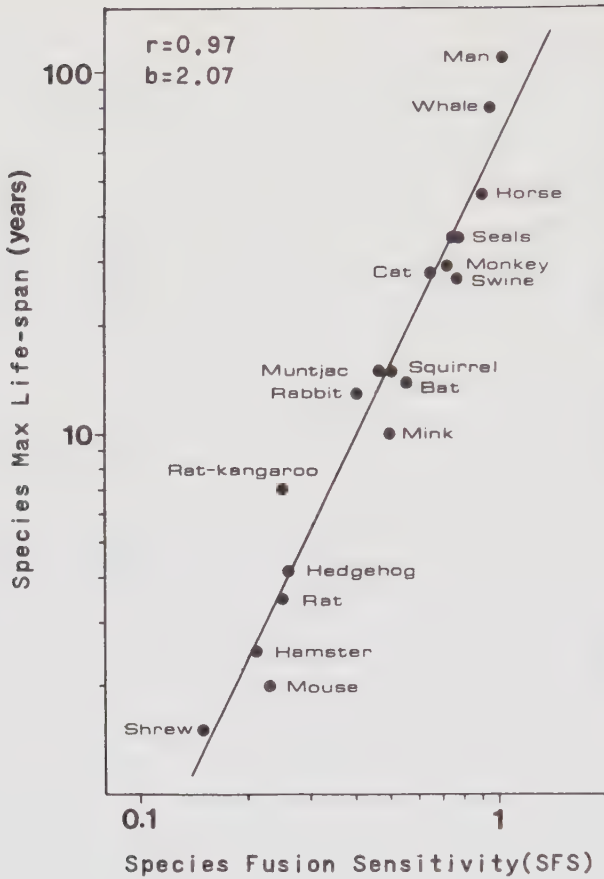


Fig. 1 Linear correlation (log/log) between the species fusion sensitivity (SFS) potentials of nineteen normal mammalian fibroblastic cultures and species maximal life-span, MLS (years) based on the data given in Table 1.

and the template activity to be decreased (see Cutler 1978). Whatever the specific mechanisms may be, it is concluded that there is a significant bulk of evidence supporting the existence of age-related changes in the structural conformation of chromosomes.

b. The Primary Structure (DNA)

That the ageing process may result from inherent instability of the genetic material was first suggested from the studies of Henshaw et al (1947). They found that animals exposed to radiation exhibit both a shortening of normal life-span and an increased incidence of age-related effects. The possible role of the genetic apparatus in the ageing process has attracted many investigators and no doubt represents one of the most extensively studied problems in the area of ageing research. During the years, the somatic mutation theory has been modified in various ways and may perhaps today rather be considered as a group of theories (Szilard 1959, Failla 1960, Orgel 1963, Curtis and Crowley 1963, Holliday and Tarrant 1972, Burnet 1974, Medvedev 1972, Cutler 1974, DeMars 1977, Ohno and Nagai 1978). The numerous observations for and against these theories are discussed at length in any current book on ageing (see Burnet 1974, Strehler 1977, Kanungo 1980). Here I will only discuss a few cases with reference to ageing in cell cultures to point out the major elements of these theories.

In the first type of experiments, the life-span of tetraploid human cells (obtained by colchicine or cell fusion) in culture was demonstrated to be the same as that of diploid cells (Hoehn et al 1975, 1978, Thompson and Holliday 1978). Secondly, in experiments with hybrid crosses long-lived x short-lived human fibroblastic cells, the life-span of the tetraploid hybrid cells was demonstrated to be intermediate to those of tetraploid parental cells (Hoehn et al 1978). In a third type of experiments, cytoplasts (enucleated cytoplasms) and karyoplasts (nucleus surrounded by a cell membrane and some residual cytoplasm) were isolated from young and old cell cultures using the method with cytochalasin B, and fused in various combinations of cytoplasts and karyoplasts to reconstitute cells (Muggleton-Harris and Hayflick 1976). The clones derived from young karyoplasts x old cytoplasts reconstitutions showed a slightly greater proliferative capacity than the opposite crosses, but far less than what was observed in the young karyoplasts x young cytoplasts. Considering the fact that the karyoplasts are surrounded with cell membrane and some residual cytoplasm, the interpretation of an intermediate proliferative capacity of the "hybrid" crosses seem likely (Norwood 1978).

The possibility that ageing of cells in culture is related to accumulation of recessive somatic mutations is not a likely explanation in neither of the three experiments discussed above. Since all cell cycle mutants so far described are recessive (Liskay and Meiss 1977, Hartwell 1978), one would expect

complementation for enhanced longevity in the tetraploid and hybrid cells. If the somatic mutations were dominant in nature, selection against these mutations would lead to a rapid elimination of effected cells from the population (DeMars 1977, Ohno and Nagai 1978) and they should therefore not be of relevance to senescence in cell cultures.

Somatic mutations might also accumulate on the basis of intrinsic differences in the fidelity of protein synthesis, as originally postulated by Orgel (1963). This theory emphasized the positive feedback effects of errors in the synthesis of proteins that themselves participate in protein synthesis, such as aminoacyl tRNA synthetases and RNA polymerases. Eventually, errors in the synthesis of DNA polymerases may be expected to occur, thus leading to somatic mutation (Holliday and Tarrant 1972). The experiments discussed above could be compatible with this error theory. In fact, disregarding the fact that it can not account for the different rates by which accumulation of errors in cells of various life-spans, I do not know of any experiment to disprove this theory. However, apart from the rate of accumulation, it is compelling that despite the extensive testing of the error theory there is as yet no proof of any protein showing changes in primary structure in old cells as would be expected (see Gallop et al 1978, Kanungo 1980). In fact, the most controversial case, the change in heat-lability of glucose-6-phosphate dehydrogenase sometimes reported in senescent cultures, appears to be due to post-synthetic modifications rather than to age-related changes in primary structure (Kahn 1977). Furthermore,

the frequency of mutant viral progeny is not increased in polio-infected terminally senescent cultures (Holland et al 1973).

A third type of somatic mutations to be considered relates to the redundant message theory (Medvedev 1972, Cutler 1974, DeMars 1977, Ohno and Nagai 1978). According to this theory, mutations may gradually accumulate in reiterated genes, such as ribosomal and tRNA cistrons without being selected against, thus setting the stage for the eventual run out of message. The fact that the number of copies of rDNA and other reiterated DNA decreases in old age (Johnson and Strehler 1972, Cutler 1978, Shmookler Reis and Goldstein 1980) may support this theory. However, the possibility that various life-spans of mammalian species would be related to the extent of redundancy of these sequences or genes, as originally postulated by Medvedev (1972) do not seem to hold true (Cutler 1974). On the other hand, Cutler (1974) found evidence that the number of active ribosomal genes was inversely related to the life-span of the species. Taken together with the results of the bobbed mutations of the *Drosophila* (see Strehler 1978), these results rather favour that the actual number of at least rDNA is secondary to the mechanism(s) regulating their activity and that there are mechanisms for deletion as well as amplification of their number operating on the basis metabolic requirements. Anyhow, with reference to the three cell culture experiments described in the beginning of this section, these results are not likely to be explained by this theory.

V. TOWARDS A MODEL

Evidence is presented to favour the concept that:

- (1) The longevity of a mammalian species is basically related to the longevity of its cells, i.e. there is an intrinsic cellular program for the regulation of life-span.
- (2) There are constitutional factors expressed in relation to the cellular life-span (at present three such factors are known). These factors are also related to the constitutional factors correlated with the longevity of the organism as a whole.
- (3) The constitutional factors related to the cellular life-span, most likely share a common regulatory control, which is suggested to be manifested in the cell membrane.

Available data on the genetic basis for longevity favour a primary role of genetic regulatory mechanisms rather than differences in structural genes. This is evidenced from evolutionary considerations as well as from studies at the level of the individual. Taking this together with the fact that the extremely diverse and complex manifestations of age are expressed with great precision in relation to the life-span, the call for a common mechanism for regulatory control is almost inevitable.

Could it be that there exists a mechanism, which involves a general interaction between the genome and the cell membrane operating in such a way that the genome specifies the composi-

tion of the cell membrane which in turn specifies the genome expression? A model based on this principle has recently been suggested for the control of cell differentiation (Brunner 1977). To emphasize the direct role of the cell membrane in specifying genome expression, the term "membrane impression" was introduced by this investigator. Since rate limiting steps in differentiation may be related to longevity, a common control for the regulation of longevity and differentiation might be expected. In fact, in a way one may regard differentiation and longevity as qualitative and quantitative aspects, respectively, of the same basic phenomenon. It is noteworthy that in these types of models, the cell membrane, by transduction of signals from the outside may act as a regulatory control point and possesses an equivalent counterpart function as the genome.

In relation to ageing, the "membrane impression" affecting the genome expression may be essentially exerted by means of its control of the ionic strength in the cell which regulates the conformational status of the chromatin (see Lezzi and Roberts 1972, MacLean and Hilder 1977, Zs. Nagy 1978). Age-related changes in membrane composition and function and their consequences for intracellular ionic strength (see paper V, Pieri et al 1977, Zs. Nagy 1978) thus lead to conformational changes in chromatin structure which will ultimately result in repression of genetic activity (Zs. Nagy 1978). The chromosome model presented (paper VIII) provides a programme for specifying the genetic expression imposed by the cell membrane. Changes in

longevity may result from structural alterations in the chromosome by means of "position effects". Particularly interesting mechanisms for providing such chromosome changes could be the "sequence interspersion" of reiterated sequences (Davidson et al 1975, 1977, Britten and Davidson 1971) and "moveable genetic elements" (see Young 1979, Temin 1980).

Lastly, the apparent immortality of cancer cells may according to the model discussed result from structural alterations of the chromosome leading to the occupation of "critical" genes to new positions where they may escape inactivation. This is provided there is a tendency of these cells to constantly generate new structural combinations. If this is true, the immortal nature of cancer cells would be related to their tendency to be regularly "rejuvenated" by this mechanism. Whether the mechanisms behind chromosome structural alterations operate on a random or non-random basis, one would expect the occurrence of senescent cells in cancer or "transformed" cell populations. Indeed, clonal attenuation of the growth potential has been observed in such cell populations (Martin et al 1975, Martinez et al 1978). Thus, at least certain genes involved in the "rejuvenation" may have to change positions whenever structural conformation imposes on them inactivation. In that way the cell can continue to replicate and thus escape from being lost. Under such conditions, the only rules to be followed are those for maintaining cellular homeostasis. The lack of obvious relations of species' life-span to evolutionary position and of finding

any rationality for positive or negative criteria in relation to longevity of the species, may suggest that these are also the rules for ageing and longevity.

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Table 1: Equations defining the relationships between species maximal life-span (MLS) and (a) replicative life-span of cultured mammalian fibroblasts, (b) the species fusion sensitivity (SFS) potential, (c) the rate of UV-induced excision repair, and (d) the rate of DMBA conversion to a proximal mutagen.

Parameter	Equation ¹⁾	No. of species studied	Correlation coefficient	t ²⁾	Reference
Fibroblast life-span (PD)	$\log_{10} \text{MLS} = 2.00 \log_{10} \text{PD} - 1.51$	9	0.948	7.88	paper I and this paper
Species fusion sensitivity (SFS)	$\log_{10} \text{MLS} = 2.07 \log_{10} \text{SFS} + 1.81$	19	0.972	17.06	paper I.
UV-induced excision repair (UDS) ³⁾	$\log_{10} \text{MLS} = 2.16 \log_{10} \text{UDS} - 1.94$	7	0.935	5.90	Hart and Setlow (1974)
DMBA conversion (K) ⁴⁾	$\log_{10} \text{MLS} = -1.00 \log_{10} \text{K} + 1.70$	6	0.979	9.60	Schwartz and Moore (1977)

- 1) All equations are calculated on a $\log_{10}/\log_{10}$ basis in all cases using the species maximal life-span (MLS) given in Biol.Data Book (1972) or else as in paper II.
- 2) Student's t-test. All parameter are significant at the level of $p < 0.001$, except UDS $p = 0.002$.
- 3) UDS= the extent of repair obtained at a UV fluence (254nm) of $10\text{J}/\text{m}^2$. Data from Fig. 4 in Hart and Setlow (1974)
- 4) K = the rate constant for the binding of DMBA to DNA (pmoles DMBA/mg DNA/hour). Data from Fig. 1 in Schwartz and Moore (1977).

Table 2: The fusion sensitivity (FS) potential and the extent of excision repair induced by UV-irradiation in human fibroblastic HES5 cells at PDL15 cultivated in two different bovine sera.¹⁾

Sera ²⁾	FS potential ³⁾ (x 10 ³)	Grains/nucleus ⁴⁾
FBS1	17.21	69.8
NCS3	10.86	46.2
Ratio $\frac{FCS1}{NCS3}$	1.58	1.51

- 1) The cells were trypsinized and incubated in medium supplemented with 15% of the serum to be tested in 24 hours before the two cell parameters were measured. (Prior to the experiment, flie cells could be cultivated in either sera effecting the result of the experiment (Röhme in prep.).)
- 2) FBS1 = fetal bovine serum, NCS3 = newborn calf serum. (These two represent particular test batches for inducing high and low value in the parameters measured and may not be typical for FBS and NCS sera in general.)
- 3) Measured according to the procedures given in paper III.
- 4) The cells were exposed with a UV fluence (254 nm) of 10J/m². The procedures were according to Hart and Setlow (1974).

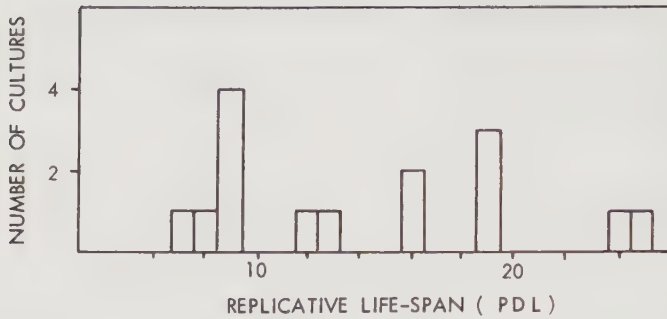


Fig. 1. Replicative life-span of 15 embryonal fibroblast cultures (whole embryos) from Syrian hamster. Cultivation procedure and calculations of life-span as in paper I.

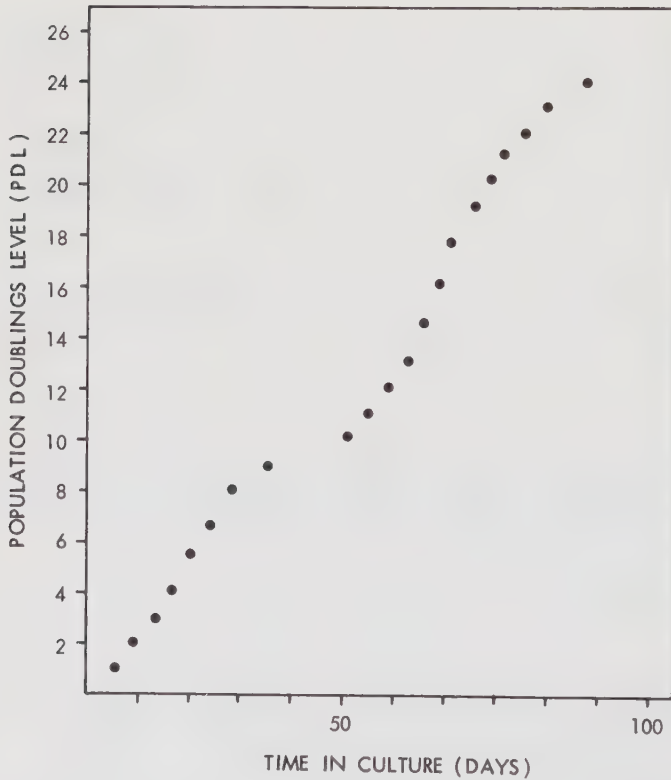


Fig. 2. Growth curve of an embryonal fibroblast culture (whole embryo) from Syrian hamster.

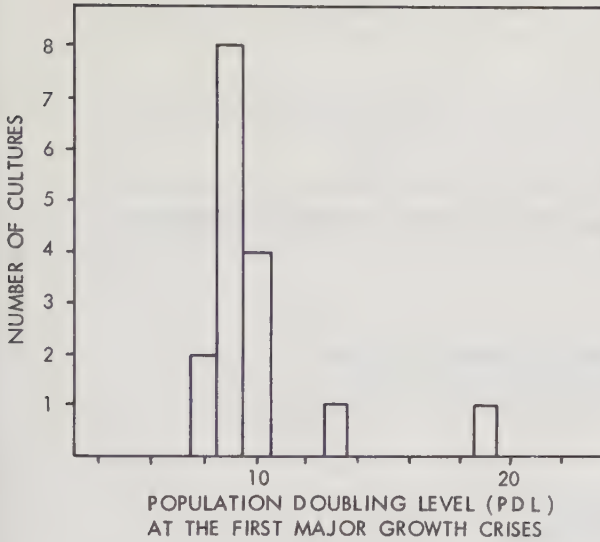


Fig. 3. Population doubling level (PDL) at the first major growth crisis occurring in 16 embryonal fibroblast cultures (whole embryos) from Syrian hamster. Cultivation procedures and calculation of PDL as in paper I.

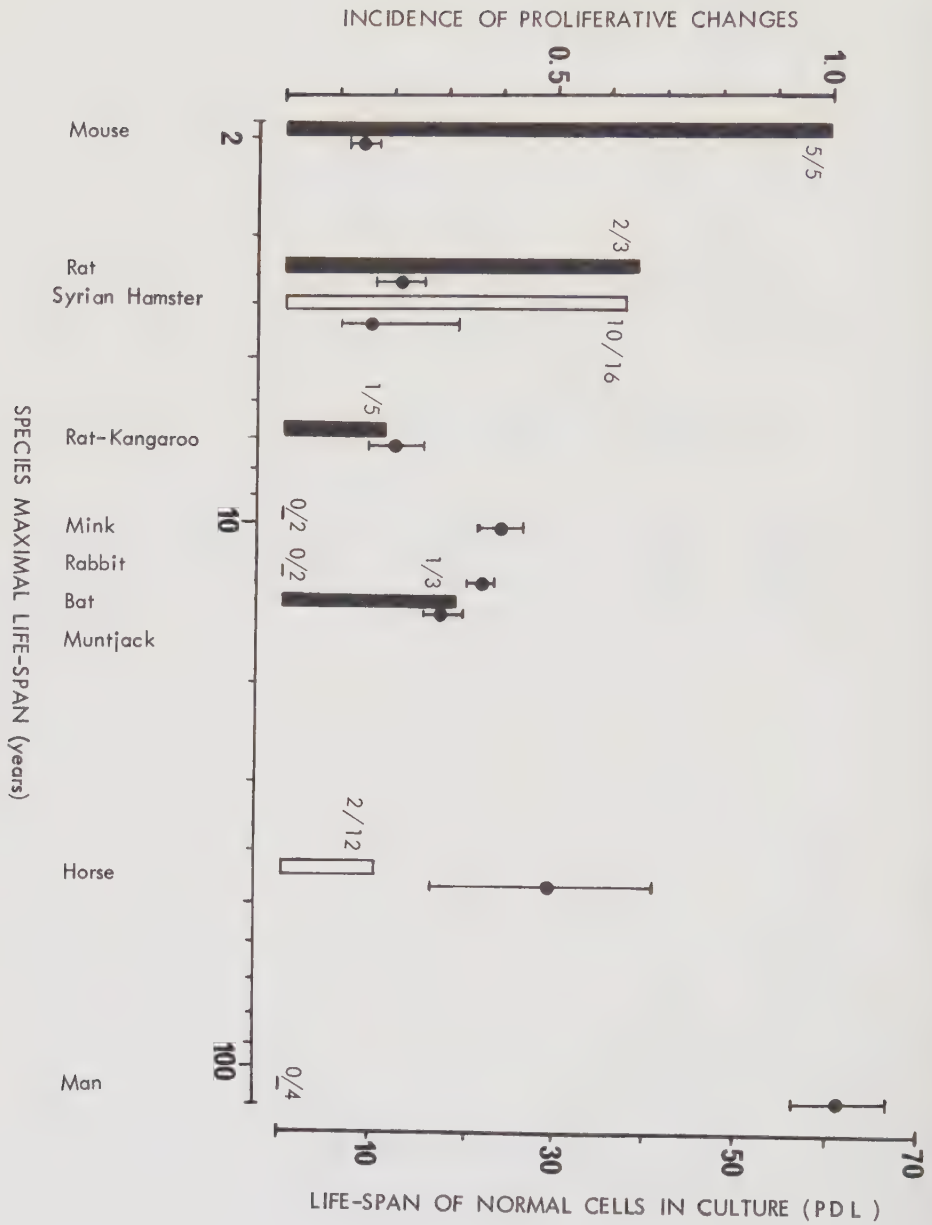


Fig. 4. The species maximal life-span related to: (a) The life-span of its cells in culture expressed as averages and ranges (right axis). The number of cultures studied for each species are the denominators in the ratio given. (b) The incidence of proliferative changes (left axis) leading to the establishment of continuous cell lines (filled rectangles) or followed by a finite life-span (unfilled rectangles). The frequencies of cultures showing these changes are given in the vicinity of the rectangles. Data from paper I and this paper.

